REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE *(DD-MM-YYYY)*	2. REPORT TYPE	3. DATES COVERED *(From - To)*
23-03-2004	Final Report	01-04-2000/31-03-2003

4. TITLE AND SUBTITLE
In-Situ Survival Mechanisms of Sulfate-Reducing Bacteria in Polluted Marine Sediments

5a. CONTRACT NUMBER
N00014-00-1-0558

5b. GRANT NUMBER

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
Q. Luo, J.L. Groh, J.D. Ballard, and L.R. Krumholz

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
The University of Oklahoma
Office of Research Services
731 Elm Avenue, Room 134
Norman, OK 73019

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)
Office of Naval Research
Attn: Linda A. Chrisey
Ballston Centre Tower One
800 North Quincy Street
Arlington, VA 22217-5660

10. SPONSOR/MONITOR'S ACRONYM(S)
ONR

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION/AVAILABILITY STATEMENT
Distribution unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

Sediment dwelling anaerobic bacteria are capable of utilizing a wide range of electron donors and acceptors, and they play a key role in the bioremediation of marine contaminants. We believe that these microorganisms have unique genes that are required only during exposure to the natural environment. Signature tagged mutagenesis (STM) has be used to identify these genes. Desulfovibrio strain G20 and Shewanella oneidensis MR-1 were studied in a model sediment system. Approximately 1-2% of all mutants screened were attenuated in sediment survival. Protein products from interrupted genes fell into several functional categories: energy production and conversion, amino acid & inorganic ion transport and metabolism, cell envelope biogenesis, cell motility and secretion, signal transduction and mechanisms, prophage functions and uncharacterized conserved proteins. These functions are believed to be crucial for sediment growth and will when fully characterized, will dramtically broaden our understanding of bacterial growth in natural systems.

15. SUBJECT TERMS
Sulfate-reducing, Mutagenesis, Survival, Contaminants

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			Lee R. Krumholz
Unclass	Unclass	Unclass	UL	4	19b. TELEPHONE NUMBER *(Include area code)* 405-325-0437

FINAL REPORT

GRANT # N00014-00-1-0558

PRINCIPAL INVESTIGATOR: Lee R. Krumholz, Jimmy D. Ballard, Joseph M. Suflita

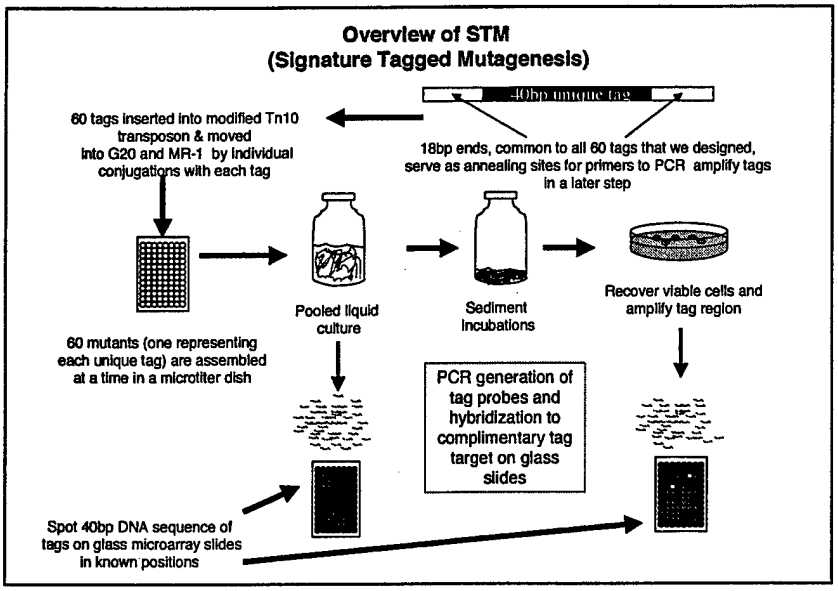
INSTITUTION: University of Oklahoma

GRANT TITLE: In Situ Survival Mechanisms of Sulfate Reducing Bacteria in Contaminated Marine Sediments.

AWARD PERIOD: 01 April 2000 – 31 March 2003.

OBJECTIVE: To identify genes needed for survival of anaerobic microorganisms in contaminated marine sediments.

APPROACH: *Desulfovibrio* strain G20 and *Shewanella oneidensis* MR-1 were used as model organisms for signature tagged mutagenesis (STM) to identify genes involved in sediment survival. Six thousand mutants of *Desulfovibrio* and 12,000 mutants of *S. oneidensis* were generated by tagged Tn10 transposons and assembled into pools of 60 mutants, representing the 60 unique tags in use. Each pool was incubated in sediments to identify potential non-surviving mutants by a microarray-adapted method we have previously developed. Strain G20 was incubated in sediments at room temperature for a 7-9 day incubation. For *Shewanella*, extensive studies were conducted to find a suitable sediment system to use for STM. Addition of lactate was found to be a minimum requirement in order for these organisms to grow and survive in Fe-reducing sediments (subsurface sediments amended with Fe(III) oxyhydroxide). *Desulfovibrio* and *Shewanella* pools were screened by reisolating cells from sediment when they grew to maximum concentration or several days following peak growth. Populations of *Desulfovibrio* increased 10 to 50-fold over their initial inoculum during the first week of incubation, and *Shewanella* increased at least 10-fold over 3 days. This growth was found necessary for selection of attenuated mutants in sediment. Using our microarray hybridization technique, approximately 3% of all mutants screened were found to be attenuated in sediment survival for both organisms. These potential non-survivors were then tested again in sediment individually or with the wild type strain in competition experiments. Of all mutants screened, ~2% of G20 and ~1% of MR-1 mutants are confirmed non-survivors. The genes interrupted by transposon mutagenesis have been identified by sequencing and comparison to genomic databases.



ACCOMPLISHMENTS: **1. RAP-PCR**

We have tested the use of RAP PCR as a means of looking at differential expression in *Desulfovibrio* sp. Cells were grown with two different electron donors, lactate and H_2. RNA was extracted and reverse transcribed under low-stringency conditions utilizing a set of random primers, and candidate cDNAs were cloned, sequenced and characterized by BLAST analysis. Putative differentially expressed transcripts were confirmed by Northern blot analysis. Interestingly, dissimilatory bisulfite reductase was upregulated in the presence of hydrogen. To link transcriptional changes to the physiology of sulfate-reducing bacteria, sulfide was measured during growth of several strains of *Desulfovibrio* on hydrogen or lactate and revealed that hydrogen-grown cells produced more sulfide per unit of cell mass than lactate-grown cells. Expression of other redox proteins was characterized by Northern blotting to determine whether or not they were also expressed to higher levels in hydrogen-grown cells. Lactate growth produced higher expression of NiFe hydrogenase. H_2 grown cells expressed the adenylylsulfate reductase b subunit and HmcA to higher levels. The results provide new insight into the continuing debate over how *Desulfovibrio* species utilize redox components to generate membrane potential and to channel electrons to sulfate (Steger et al., 2002) .

More recently, a study was undertaken with *D. vulgaris* to characterize genes whose expression was increased in the presence of toxic metals (Cu or Hg;(Chang *et al.*, 2003; Chang *et al.*, 2004)). Cells were grown under conditions in which growth rate was partially inhibited (50 μM of either metal) and expression profiles were determined using RAP-PCR. The genes for ATP binding protein (multi-drug resistance protein) and an ATPase (ORF 856) were upregulated (4-6 fold with Hg and 1.4 to 3 fold with Cu) in metal treated cultures, suggesting that *D. vulgaris* uses an ATP dependent mechanism (perhaps as an efflux pump) for adapting to toxic metals in the environment.

2. Signature Tagged Mutagenesis. Genes identified to be required for sediment survival are summarized below in Tables 1&2.

Table 1. Genes required for survival in *Desulfovibrio* G20. Genome is currently being annotated and we therefore are not able to assign specific genes.

Mutant	Product	Functional (COG) Category
B8(pE1)	ABC-type proline/glycine betaine transport systems, periplasmic components	Amino acid transport and metabolism (E)
G9(pB1)	ABC-type polar amino acid transport system, ATPase component	Amino acid transport and metabolism (E)
B2(pF1)	D-alanyl-D-alanine dipeptidase	Cell envelope biogenesis, outer membrane (M)
A6(pA2)	N-acetylmuramoyl-L-alanine amidase	Cell envelope biogenesis, outer membrane (M)
D5(pD2)	methyl-accepting chemotaxis protein	Cell motility and secretion (N)
B2(pG1)	chemotaxis protein; stimulates methylation of MCP proteins	Cell motility; Signal transduction (N,T)
D5(pB3)	succinyl-CoA synthetase, beta subunit	Energy production and conversion (C)
G9(pC1)	pyruvate:ferredoxin oxidoreductase and related 2-oxoacid:ferredoxin oxidoreductases, alpha subunit	Energy production and conversion (C)
D5(pC2)	hypothetical protein	Function unknown (S)
B11(pF2)	uncharacterized conserved protein	Function unknown (S)
B11(pC2)	predicted membrane-associated HD superfamily hydrolase	General function prediction only (R)
A3(pE2)	predicted permeases	General function prediction only (R)
H8(pE2)	trans-aconitate methyltransferase	Metabolism
C12(pG2)	phage-related tail protein	Prophage functions

Table 2. Genes required for survival in *Shewanella* MR-1. We also obtained 4 conserved hypothetical proteins and 2 putative hypothetical proteins.

Mutant	*InvivoCI**	Gene	Product	TIGR cellular role	COG
A12mB12	0.0031	*ccm*F-2	Cyt. c-type biogenesis protein CcmF	Energy metabolism; electron transport	O
A10mB4	0.054	*pho*B	PO4 regulon response regulator PhoB	Two-component systems	T,K
B10mC10	0.106	*rec*G	ATP-dependent DNA helicase RecG	DNA replic., recomb., & repair	L,K
A10mA9	0.011**		phosphate acetyltransferase	Electron transport & fermentation	C
B2mA1	0.28**	*gsp*N	General secretion pathway protein N	Protein and peptide secretion and trafficking	
B9mG5	0.282		TonB-dep. receptor C-term. domain protein	Unknown function: general	P
C2mD10	0.315	*cys*Q-1	cysQ protein	Central intermediary/sulfur metabolism	P,G
B4mC10	0.53	*moe*A	molybdopterin biosynthesis MoeA protein	Molybdopterin biosynthesis	H
B5mG9	0.59		HlyD family secretion protein	Transport and binding proteins	R
C2mB9	0.59	*hyd*A	periplasmic Fe hydrogenase, large subunit	Energy metabolism: Electron transport	C
B12mD10	0.277	*mex*F	RND multidrug efflux transporter MexF	Toxin prod. and resistance; Transport and binding proteins: Unknown substrate	P
E3mA8	0.302		transcriptional regulator, TetR family	Regulatory functions; DNA interactions	K
C4mA5	0.155	*ilv*Y	transcriptional regulator IlvY	Regulatory functions; DNA interactions	K
B11mC7	0.061**	*arg*G	argininosuccinate synthase	Amino acid biosynthesis: glutamate	E
D3mG7	0.036**	*arg*H	argininosuccinate lyase	amino acid biosynthesis-glutamate family	E
D5mC5	0.006	*glt*D	glutamate synthase, small subunit	Amino acid biosynthesis: glutamate	E,R
B12mE2	0.706		Prophage MuSo2, tail sheath protein	Prophage functions	
C9mA10	0.073**	*arg*H	argininosuccinate lyase	amino acid biosynthesis-glutamate family	E
E7mD4	0.232**	*gap*A-3	glyceraldehyde 3-phosphate dehydrogenase	Glycolysis/gluconeogenesis	G
A4mD11	0.594		serine protease, subtilase family	Protein Degradation	O
D7mA4	0.145	*leu*A	2-isopropylmalate synthase	Amino acid biosynthesis: pyruvate family	E
E2mG11	0.548		ISSod4, transposase	Transposon functions	

* *in vivo* CI=sediment microcosms

**=growth in lactate-Fe(III)citrate media is impaired (CI<0.5)

COG functional groups. C=energy production and conversion; E=amino acid transport and metabolism; G=carbohydrate transport and metabolism; H=coenzyme metabolism; K=transcription; L= DNA replication, recombination and repair; O= Posttranslational modification, protein turnover, chaperones; P=Inorganic ion transport and metabolism; R=general fxn prediction only; T=signal transduction mechanisms.

Discussion of a Select Group of Mutants.

We have identified several genes that are important for sediment survival of G20 and MR-1. MR-1 genome analysis indicates that it has 39 c-type cytochromes, including 14 with four or more heme-binding sites that have not been described before. The *ccm*F gene interrupted in our studies is one of these 14 cytochromes (SO0478 locus on TIGR *Shewanella* genome page) and is required for sediment survival only (not required for growth in Fe(III)-citrate media). The MR-1 genome sequence also contains the first reported heterodimeric hydrogenase enzyme family in a facultative aerobe (SO3920 and SO3921-large and small subunits, respectively). Gene locus SO3920 was interrupted in MR-1. This gene is most similar to the periplasmic hydrogenase in *D. vulgaris* which has been implicated in hydrogen uptake for electron transfer to low-potential, multiheme c-type cytochromes which may be involved in metal reduction. Regarding RecG, its overexpression in previous work, conferred on *E. coli*, an increased resistance to several weak organic acids, including acetate. Similarly, this intact gene in MR-1 may confer resistance to organic acids present in the sediment. Interestingly, in both G20 and MR1, phage genes affect sediment survivability: phage related tail protein in G20 and prophage MuSo2 putative tail sheath protein in MR-1. Interruption of methyl-accepting chemotaxis protein (MCP) genes caused G20 to lose the ability to survive in the sediment. Previous work has shown that a methyl-accepting protein, DcrA from *D. vulgaris* is thought to sense oxygen concentration or redox potential of the environment. However, the two MCP genes interrupted in our study have low similarity to the *D. vulgaris* DcrA. These genes may serve a similar function, though as sensors responding to redox stress or they may be involved in sensing the presence of nutrients in the environment. Two ABC transporter genes were also important for sediment survival of G20. ABC transporters use the energy associated with ATP hydrolysis to translocate a wide variety of solutes across cellular membranes. These molecular pumps are found in all phyla and form one of the largest of all families. These pumps could be used for one or more of a variety of functions including detoxification of metals, release of antibiotics, etc. We have recently reported that *D. vulgaris* increased expression of two different ATP dependent transporters in response to Hg^{2+} and Cu^{2+} stress (Chang *et al.*, 2004).

SIGNIFICANCE: These studies are providing useful information on cellular functions needed for sediment survival. This information may ultimately aid us stimulating bioremediation of contaminants of sediment systems.

PUBLICATIONS AND MAJOR ABSTRACTS:

1. Chang, I.S., J. L. Steger, M. M. Ramsey, J.D. Ballard and L.R. Krumholz. 2004. Differential Expression of *Desulfovibrio vulgaris* genes in response to Cu(II) and Hg(II) toxicity. **Appl. Environ. Microbiol.** 70:1847-1851.
2. Chang, I., J.D. Ballard and L.R. Krumholz. 2003. Evidence for Chimeric sequences during random arbitrarily primed PCR. **J. Microbiol Meth.** 54:427-431.
3. Steger, J.L., C. Vincent, J.D. Ballard and L.R. Krumholz. 2002. *Desulfovibrio* sp. Genes involved in the metabolism of Hydrogen and Lactate. **Appl. Environ. Microbiol.** 68(4):1932-1937.
4. Luo, Q., J.L.Steger, D.J. Smalley, J.D. Ballard and L.R. Krumholz. 2003. A new screening system with microarray technology for signature tagged mutagenesis with *Desulfovibrio* and *Shewanella.* American Society for Microbiology 103rd General Meeting. Q-362.
5. Differential expression of Desulfovibrio sp. genes during growth on hydrogen versus lactate. 2001. J.L. Steger, C. Vincent, L.Krumholz and J. Ballard. In *Abstracts of the 98th General Meeting of the American Society for Microbiology.* N-96.